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A gain-of-function mutation in *Msl10* triggers cell death and wound-induced hyperaccumulation of jasmonic acid in *Arabidopsis*

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Research Article

Abstract Jasmonates (JAs) are rapidly induced after wounding and act as key regulators for wound induced signaling pathway. However, what perceives the wound signal and how that triggers JA biosynthesis remains poorly understood. To identify components involved in *Arabidopsis* wound and JA signaling pathway, we screened for mutants with abnormal expression of a luciferase reporter, which is under the control of a wound-responsive promoter of an ethylene response factor (*ERF*) transcription factor gene, *RAP2.6* (Related to APetala 2.6). The *rea1* (RAP2.6 expresser in shoot apex) mutant constitutively expressed the RAP2.6-LUC reporter gene in young leaves. Along with the typical JA phenotypes including shorter petioles, loss of apical dominance, accumulation of anthocyanin pigments and constitutive expression of JA response gene, *rea1* plants also displayed cell death and accumulated high levels of JA in response to wounding. The phenotype of *rea1* mutant is caused by a gain-of-function mutation in the C-terminus of a mechanosensitive ion channel *MscS-like 10* (*MSL10*). MSL10 is localized in the plasma membrane and is expressed predominantly in root tip, shoot apex and vascular tissues. These results suggest that MSL10 is involved in the wound-triggered early signal transduction pathway and possibly in regulating the positive feedback synthesis of JA.

Keywords: *Arabidopsis*; cell death; jasmonates; mechanosensitive ion channel; wound
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INTRODUCTION

Plants are subjected to many mechanical stresses. Upon wounding mobilization of specialized cells for healing as a protective measure is not a possibility since plants do not have a circulatory system. Instead, plants have developed inducible defense mechanisms that are specific to different stresses. The phytohormone jasmonic acid is a well characterized signaling molecule that is widely accepted for its role in protection against the wound damage (Leon et al. 2001). Because of the similarity in the nature of damage caused by mechanical damage and herbivore feeding, plants utilize jasmonic acid pathway for protection against both. In addition to their role in plant defense, jasmonates are also required for pollen development, anther dehiscence, protection against ozone damage and inhibition of root elongation (Rao et al. 2000; Ito et al. 2007; Chen et al. 2011; Song et al. 2011).

Endogenous JA levels increase in response to mechanical wounding, herbivore damage and water stress (Creelman and Mullet 1997; Rao et al. 2000; Diezel et al. 2011). Biosynthesis of JA occurs through the octodecanoid pathway and is initiated by the addition of linolenic acid (18:3) through lipoxygenase (LOX) to form 13-hydroperoxylinolenic acid. Then it is dehydrated by allene oxide synthase (AOS) and cyclized by allene oxide cyclase (AOC) to the cyclopentanone 12-oxo-phytodienoic acid (OPDA). The pentacyclic ring double bond in OPDA is reduced by the enzyme OPDA reductase 3 (OPR3) to form OPC: 8, which is subsequently shortened by three cycles of β-oxidation to yield JA (Li et al. 2005). JA, its precursor OPDA and their related oxylipins are collectively called jasmonates. The synthesized JA can be converted into jasmonoyl-L-isoleucine (JA-Ile), an active form of jasmonate (Fonseca et al. 2009). Upon binding to its receptor COI1 (Yan et al. 2009; Sheard et al. 2010), it promotes the degradation of jasmonate ZIM-domain (JAZ) family of transcriptional repressors thereby promoting the activation of jasmonate response genes (Chini et al. 2007; Thines et al. 2007; Staswick 2008).

Because of its relevance to different plant stress responses many mutant screens have been conducted to identify components involved in JA signaling. Among the identified mutants, coronatine insensitive 1 (*coi1*) is fully insensitive to JA and *Coi1* encodes an F-box protein (Xie et al.

1998). Several other JA insensitive mutants have also been characterized that have a weaker phenotype when compared to *coi1* mutant, including *jar1* (Staswick et al. 1992), *jin 1* and *jin 4* (methyl-jasmonate insensitive; Berger et al. 1996), and *jue1*, *jue 2* and *jue 3* (Jensen et al. 2002). Mutants with constitutive or enhanced JA responses have also been characterized, which include *cev1* (constitutive expression of vsp1), *cex1*, *cet1*, *fou2* and *joe1* (Ellis and Turner 2001; Hilpert et al. 2001; Xu et al. 2001; Jensen et al. 2002; Bonaventure et al. 2007). JA-Ile is perceived by COI1-JAZ receptor complex and this process can be potentiated by inositol pentakisphosphate (IP_5) (Chini et al. 2007; Thines et al. 2007; Yan et al. 2009; Sheard et al. 2010).

Studies from the expression analysis in the JA biosynthetic mutant *opr3*, which cannot synthesize JA from its precursor OPDA showed that both JA and OPDA regulate distinct sets of wound response genes. Unlike JA, the OPDA regulated genes (ORGs) do not require the signaling component COI1 for their activation (Taki et al. 2005). These results comprehensively show that the OPDA-dependent signaling pathway is required for wound mediated responses. Suza and Staswick (2008) further showed that although the JA derivative JA-Ile is required for the activation of the JA responsive genes and for defense against various pathogens, it is not required for the activation of wound-induced genes.

Mechanosensitive ion channels are believed to be the sensor for mechanical stimuli, osmotic stresses or gravity in plants. There are 10 MscS-like (MSL) proteins in the *Arabidopsis* genome. They are homologous to the most studied mechanosensitive ion channel of small conductance (MscS) in *Escherichia coli*. MscS was crystallized and shown to be gated by the membrane tension to release osmolytes. Therefore the cellular turgor could be maintained at the safe level to protect cells against the hypoosmotic shock (Levina et al. 1999). MscS channels are heptamer with three transmembrane (TM) helices at the N-terminus of each monomer. The third TM helices form the pore to conduit the release of the osmolytes (Bass et al. 2002; Wang et al. 2008). Similar to MscS, *Arabidopsis* MSL2 and MSL3 were localized in plastid and required for the proper size, shape, division, and hypoosmotic volume control of plastids (Haswell and Meyerowitz 2006; Wilson et al. 2011; Veley et al. 2012). MSL10 was also localized in plasma membrane and demonstrated to be a functional mechanosensitive ion channel with a preference for anions. However, the physiological roles of MSL10 remain unknown since *msl10* mutants responded to mechanical and osmotic stresses normally (Haswell et al. 2008; Maksaev and Haswell 2012).

The pathway involved in the synthesis of JA and its downstream signaling genes are well studied, but the components involved in initial wound signal perception and transmission leading to the synthesis of JA is not clear. The activation of JA synthesis genes upon wounding and application of JA suggests the involvement of a positive feedback control mechanism in JA synthesis, which is completely unknown. In this study we have used *RAP2.6*, a rapidly and highly induced gene in response to wounding, whose expression requires an intact JA pathway (He et al. 2004), as a marker to gain insight on the early events of the wound inducible pathway. Here, we describe the identification and characterization of a mechanosensitive ion channel involved in early wound signaling. Along with its key regulatory role in positive feedback biosynthesis of JA, it also controls other pathways involving cell death and apical dominance whose most probable regulation is upstream of JA accumulation.

RESULTS

Isolation of novel *Arabidopsis* mutant with constitutive *RAP2.6* gene expression and a loss of apical dominance phenotype

To identify the components involved in wound signal perception and its JA-dependent signaling pathway, we screened EMS-mutagenized *Arabidopsis* plants for mutants with abnormal expression of *RAP2.6-LUC* reporter transgene. Four- to 6-week-old M2 plants were sprayed with luciferin and luminance was measured. Of the 16,000 M2 plants screened, only one mutant showed constitutive *RAP2.6-LUC* expression and was thus named *rea1* (Figure 1A, B). The *rea1* phenotype was further confirmed in the M3 generation. The luciferase activity in the *rea1* mutant is 55–60 fold higher in the young and developing leaves compared to wild-type plants. However, *RAP2.6- LUC* expression in mature leaves of *rea1* is only 10–15 fold higher than wild-type plants (Figure 1C). Young seedlings of *rea1* are identical to wild-type plants in morphology except for the accumulation of anthocyanin in young and developing leaves. However, adult plants exhibited several morphological abnormalities including stunted growth, shorter petioles and loss of apical dominance (Figure 1D). During bolting, the phenotype of *rea1* is clear as the growth inhibitory effect of the main shoot on the auxillary shoots is clearly lost, giving rise to a bushy phenotype (Figure 1D). Further characterization of the mutant showed that *rea1* also has a cell death phenotype. A microscopic cell death phenotype is clearly visualized in *rea1* using trypan blue staining (Figure 1E).

rea1 accumulates higher JA levels

Accumulation of anthocyanin pigments and shorter petioles observed in rea1 plants is a characteristic feature of JA-hyperaccumulation in plants (Turner et al. 2002); we therefore examined the expression levels of JA biosynthetic and response genes. RNA- blot analysis showed that the constitutive transcript levels of the JA biosynthetic gene *LOX2* and the response gene *PDF1.2* were elevated in *rea1* as compared to the wild-type plants. In addition, wounding further induced the expression of *LOX2* in rea1 mutant. The constitutive level of another JA biosynthetic gene *AOS* is similar to the wild-type, but is induced to a higher level after wounding (Figure 2A).

Constitutive expression of JA biosynthetic and responsive genes prompted us to quantify the levels of JA and its precursor oxo-phytodienoic acid (OPDA). The levels JA or OPDA were not significantly different between the unwounded *rea1* and wild-type plants. However, wounded *rea1* plants accumulated 28 fold more JA (Figure 2B) and 10 fold higher OPDA (Figure 2C) than in wild-type plants suggesting that wound induced hyperaccumulation of JA in *rea1* mutants.

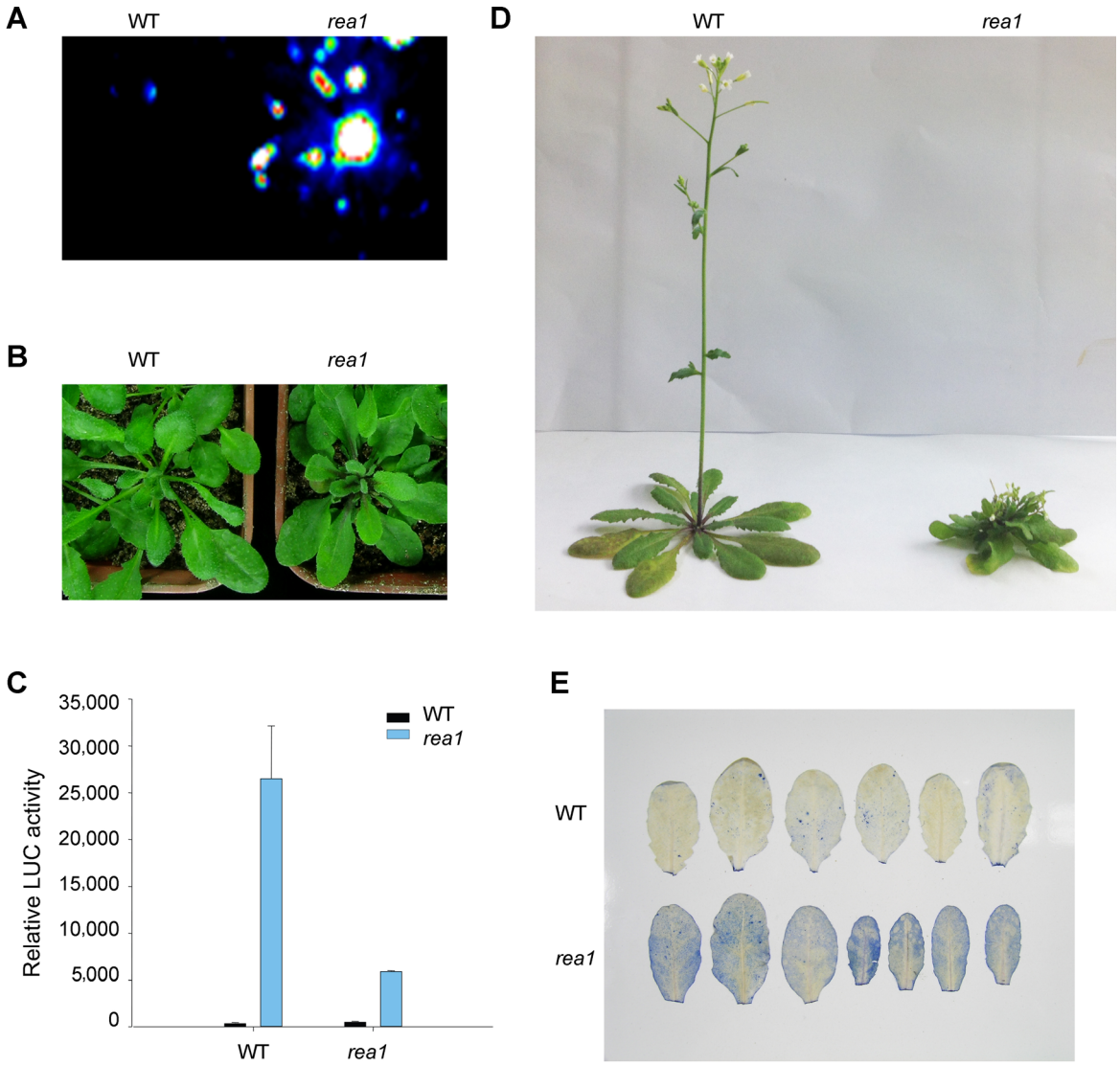

Figure 1. Phenotypes of *rea1* mutant plants
(A) *rea1* mutant plants constitutively expressing RAP2.6-LUC reporter transgene. Wild-type (left) and *rea1* (right) plants were sprayed with luciferin solution to reveal the luciferase activity. **(B)** Leaf petiole and shoot tip morphology of 4-week old wt (left) and *rea1* (right) plants. **(C)** RAP2.6-LUC activity in young and mature leaves of *rea1*. Leaves of wt and *rea1* plants were harvested and incubated with luciferin and relative luciferase activity was analyzed by using a luminometer. **(D)** Floral morphology of 5-week-old wt and *rea1* plants. **(E)** Spontaneous cell death phenotype of *rea1* mutant. Untreated leaves were stained with trypan blue to visualize cell death in the leaf tissue.

The mutant phenotype of *rea1* is partially dependent on COI1

Constitutive expression of JA related genes and wound induced hyperaccumulation of JA levels raised the possibility that the observed morphological abnormality of *rea1* could be dependent on the JA signaling pathway. To assess this possibility, we crossed *rea1* into *coi1-1*, which is required for most JA-mediated responses (Feys et al. 1994). The *rea1*; *coi1-1* double mutants did not have shorter petioles like the *rea1* mutant and the cell death phenotype was completely suppressed (Figure 3A, C). The double mutants also did not accumulate anthocyanin pigments in the shoot tip. However, the *rea1*; *coi1-1* double mutants did not completely lose their bushy phenotype (Figure 3B). The intermediate phenotype of the double mutants suggest that JA is required for anthocyanin accumulation and cell death in *rea1*, but only partially accounts for the loss of apical dominance phenotype of *rea1*.

Genetic analysis and cloning of *rea1*

To determine the nature of the *rea1* mutation, M3 mutant plants of *rea1* were backcrossed to the wild type *RAP2.6-LUC* plants. The resulting F1 plants showed a wild-type phenotype, suggesting that the mutation is recessive. Analysis of the resulting F2 individuals showed that both the constitutive luciferase expression and the bushy phenotype co-segregated

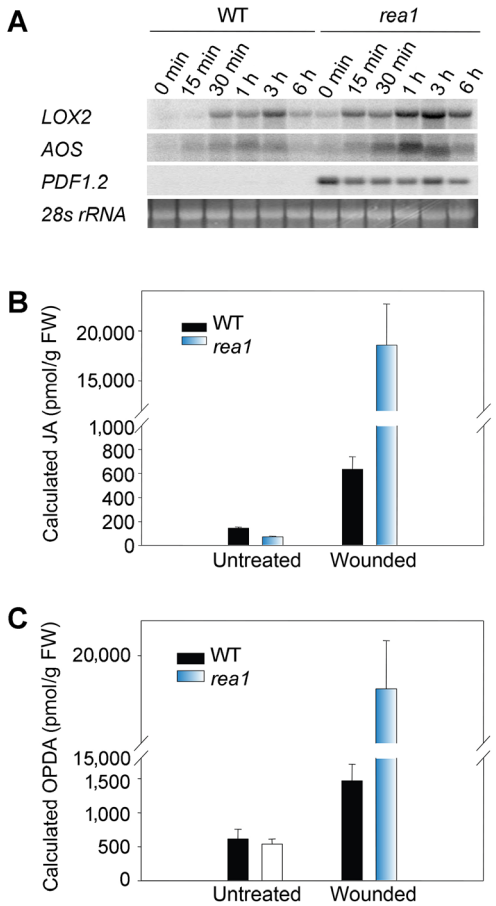

Figure 2. *rea1* plants show elevated levels of jasmonic acid (JA) accumulation after wounding

(A) RNA gel blot analysis of JA biosynthesis genes *LOX2* and *AOS* and JA response gene *PDF1.2*. Total RNA was extracted from the wounded plants and 10 μg of total RNA was electrophoresed, blotted and probed with respective probes. **(B)** Levels of JA after wounding. **(C)** Levels of 12-oxo-phytodienoic acid (OPDA) after wounding. Plants were wounded with serrated forceps and tissue was harvested after 8 h and the samples were assayed for both JA and OPDA levels. The experiments were repeated three times with similar JA levels.

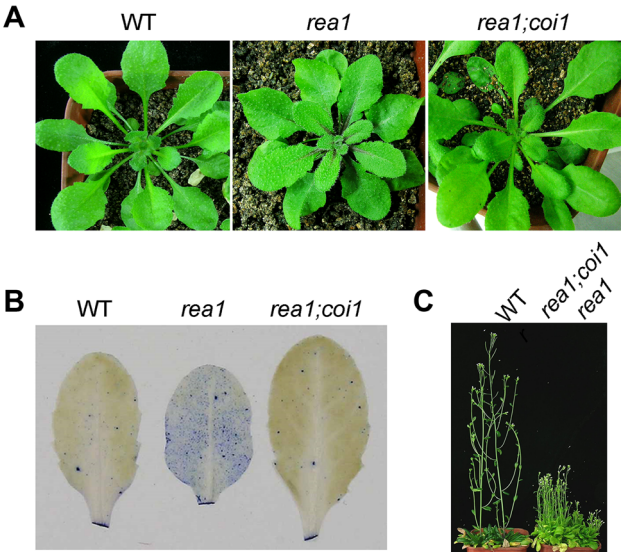

Figure 3. Phenotypes of *rea1* mutant in *coi1-1* mutant background

Leaf petiole and shoot tip morphology (**A**), cell death (**B**) and floral phenotypes (**C**) in *rea1*; *coi1-1* double mutants.

with one another and segregated in a 3 wild-type: 1 mutant fashion (Table S1), indicating that both phenotypes are controlled by a single nuclear gene. To genetically map position of the *rea1* locus, we out-crossed *rea1* mutants in Col-0 background with both Nossen and Landserg *erecta* ecotypes. As expected, the F2 progeny segregated in a 3:1 ratio for wild-type and bushy phenotypes, respectively. Thirty-five (35) *rea1* homozygous individuals derived from the F2 progeny were pooled and genotyped using 26 microsatellite markers (Bell and Ecker 1994) covering all five chromosomes of *Arabidopsis*. The *rea1* mutation was mapped to the top arm of chromosome 5 between the markers nga225 and nga106. By analyzing additional F2 plants exhibiting the bushy phenotype with newly developed simple sequence length polymorphism (SSLP) and cleaved amplified polymorphic sequence (CAPS) markers, *rea1* was positioned between the F14F18.12 and MXC9.11 regions of the BACs (bacterial artificial chromosomes) (Figure S1A). Sequencing of all 23 coding genes within this region revealed a C-to-T transition in the coding region of *Msl10* gene, resulting in a serine to leucine substitution at residue 640 in the carboxy-terminus of MSL10 (Figure S1B, 1C).

To further confirm that a mutation in the *MSL10* gene is responsible for the *rea1* phenotype, *rea1* mutant plants were transformed with a 6.3 Kb genomic fragment carrying the wild-type MSL10 gene under the regulation of its own promoter. We obtained more than 60 transgenic lines. Among them, 51 transgenic lines showed complete complementation and the plants were morphologically indistinguishable from the wild-type plants (Figure 4B), 11 plants showed partial complementation as indicated by the semi-bushy phenotype, five plants showed complete *rea1* mutant phenotype. In addition, the complemented transgenic plants had wild-type levels of expression of *RAP2.6-LUC* reporter gene (Figure 4A). And the expression of the JA synthesis and responsive genes was almost restored to wild-type levels although *LOX2* gene expression seems to be slightly elevated in the complemented line compared to the wild-type plants (Figure 4C). This may be indicative of the residual activity of the *rea1* mutant gene. Taken together, these results further confirm that the mutation in *MSL10* is responsible for the *rea1* phenotype and we assign *msl10-3* allele to the *rea1* mutation.

msl10-3 is a gain of function mutation

Compared to the wild-type plants, *MSL10* is transcribed slightly higher in *rea1* mutants as detected by quantitative polymerase chain reaction (qPCR) (Figure S2). To investigate whether the phenotype of *rea1* is a result of a non-functional allele of the *MSL10* gene, two independent T-DNA insertion lines *msl10-*

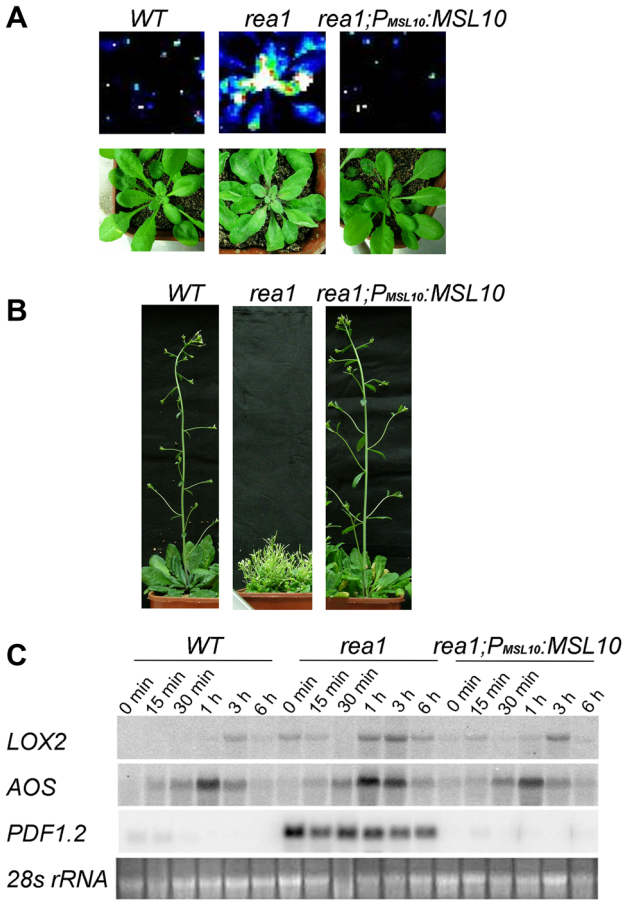

Figure 4. Complementation analysis of *rea1*
(A) A pseudo-color luminescence image (top) and the bright field image (bottom) of the untreated wild-type, *rea1* and *rea1* mutant plants transformed with wild-type copy of *MSL10* plants were sprayed with luciferin solution to reveal the luciferase activity. **(B)** Wild-type copy of *MSL10* regulated under its native promoter rescued the phenotype of rea1 mutant. **(C)** Wild-type, *rea1* and homozygous lines derived from *rea1* transformed with wild type copy of *MSL10* were wounded with a forceps and were then analyzed for the expression of JA biosynthesis and response genes.

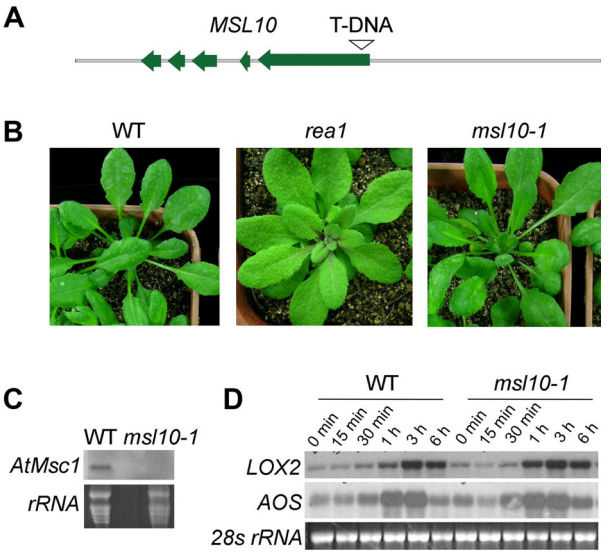

Figure 5. Phenotype of *msl10-1* mutant plants
(A) Illustration of insertion in *MSL10*. Boxes indicate the coding region of *MSL10* gene and the position of T-DNA insertion is indicated. **(B)** Morphological phenotype of 4-week-old *msl10-1* plants. **(C)** RNA gel blot analysis showing the lack of *MSL10* transcript in *msl10-1* mutant plants. **(D)** Accumulation of *LOX2* and *AOS* mRNA in wounded wild-type and *msl10-1* mutant plants.

1 (Salk_076254) and *msl10-2* (Salk_002505) containing T-DNA insertions (Alonso et al. 2003) in exon-1 and exon-5 of *MSL10* were analyzed. As both insertional mutants have similar phenotype, data from *msl10-1* are presented here. Because the T-DNA was inserted at the beginning of open reading frame (Figure 5A), the insertion is expected to give rise to a null mutant. RNA-blot analysis did not detect any transcripts of *MSL10* in both the lines confirming that the T-DNA insertion had knocked out the gene (Figure 5C). Interestingly, the morphology of the T-DNA knockout lines is similar to the wild-type plants. Neither the accumulation of anthocyanin pigment nor the loss of apical dominance was seen in the knockout mutants (Figure 5B), consistent with the previous report that *msl10-1* has no observable phenotype (Haswell et al. 2008). Besides, the expression levels of *LOX2* and *AOS* in the *msl10-1* mutant are similar to the wild-type levels, further confirming that the phenotype of *rea1* is not a result of the loss- of-function allele of *MSL10* (Figure 5D).

To further check if increased accumulation of *MSL10* transcripts could lead to the *rea1* phenotype, both the wild-type *MSL10* and *MSL10*^{S640L} were overexpressed in wild-type Col-0 plants. Plants overexpressing *MSL10* accumulated higher transcript levels, but do not have any abnormal phenotype (Figure 6A). Consistent with this result, plants accumulating higher *MSL10* transcript levels do not accumulate elevated levels of *LOX2* and *AOS* transcripts compared to the wild-type plants (Figure 6E). However, plants overexpressing *MSL10*^{S640L} transcripts demonstrate a heavier *rea1* phenotype accompanied by severe cell death (Figure 6A). 32 of 70 transgenic lines overexpressing *MSL10*^{S640L} displayed severe necrosis in leaves, and 66 of 70 lines showed severely stunted growth phenotype. Taken together, these results suggest that the observed phenotype of *rea1* is because of a gain-of-function mutation in the C terminus of MSL10 protein.

The carboxy-terminus of MSL10 is critical for its function and membrane localization

To further study the subcellular localization of MSL10, we generated transgenic lines carrying *MSL10-GFP* and *MSL10-FLAG* under the control of an estrogen-inducible promoter in both wild-type and *msl10* backgrounds. However, estradiol-induced expression of *MSL10-GFP*, but not green fluorescent protein (GFP) alone, in both wild-type and *msl10* backgrounds resulted in a phenotype similar to *rea1* (Figure 6B). In addition, plants expressing *MSL10-GFP* in the presence of estradiol also accumulated higher levels of *LOX2* and *AOS* transcript levels similar to that of *rea1* (Figure 6E). These results indicate that

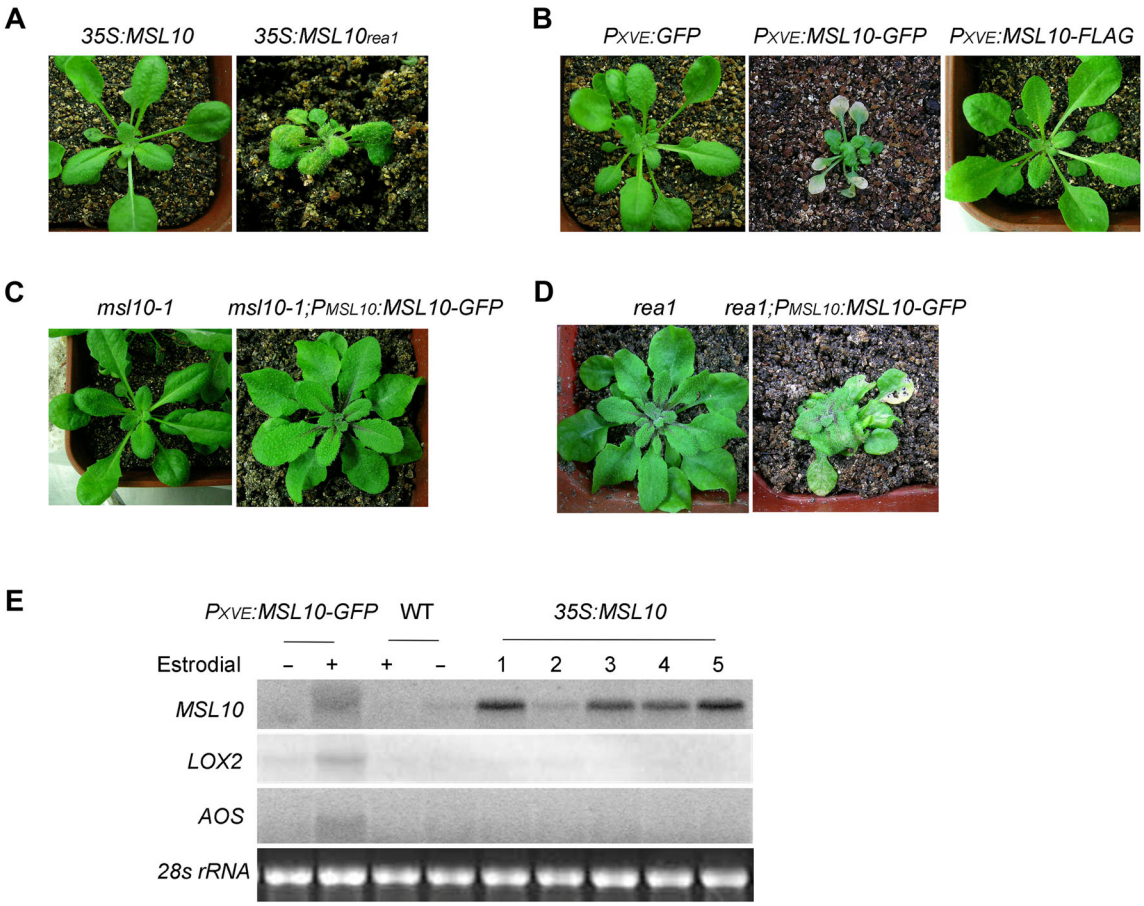

Figure 6. Plants carrying *MSL10-GFP* transgene phenocopy *rea1* mutation
(A) Overexpression of $MSL10_{rea1}$ in wild-type (WT) shows heavier *rea1* phenotype. Phenotype of plants carrying wild-type copy of *MSL10* and $MSL10_{rea1}$ regulated under the control of 35S promoter. **(B)** Estradiol-induced expression of *MSL10-GFP* in wild-type plants results in rea1 phenotype. Plants carrying empty green fluorescent protein (GFP), GFP and FLAG tagged *MSL10* under the control of estradiol were sprayed with 50 μM estradiol and the phenotypes were observed. **(C)** Phenotype of *msl10-1* plants carrying *MSL10-GFP*. *msl10-1* mutant plants were transformed with GFP tagged *MSL10* regulated under the control of its native promoter and photographs were taken after 4 weeks. **(D)** Phenotype of *rea1* plants carrying *MSL10-GFP*. Phenotype of homozygous *rea1* plants transformed with *MSL-GFP* construct. **(E)** RNA gel blot analysis showing the expression of JA biosynthesis genes in estradiol inducible *MSL10-GFP* overexpression and constitutive *MSL10-GFP* overexpression plants.

the presence of S640L mutation or GFP at the carboxy-terminus of the MSL10 most likely interferes with its normal function. However, estradiol induced expression of MSL10-FLAG in wild-type plants did not have any phenotype, suggesting that FLAG, being a very small tag compared to GFP, did not perturb the function of MSL10 (Figure 6B).

To determine the intracellular localization of MSL10 and to confirm whether *rea1*-MSL10 is similarly localized, protoplasts were prepared from the wild-type *Arabidopsis* plants and transient transformation experiments were performed. In protoplasts expressing MSL10-GFP, the signal appears to be localized mainly in the plasma membrane, which is similar to the subcellular expression patterns analyzed in MSL10-GFP transgenic plants (Haswell et al. 2008; Veley et al. 2014). Consistent with this, protein fractionation experiment showed that MSL10-GFP was exclusively present in the microsome faction (data not shown). Similarly, substantial localization of $MSL10^{S640L}$-GFP was also observed in the plasma membrane, suggesting that the mutation in MSL10 did not interfere with the localization or the stability of the protein (Figure S3). To further analyze the role of the carboxy-terminus of MSL10 protein in localization, we generated a truncated MSL10-GFP fusion construct for which the MSL10 sequences after amino acid 640 were eliminated. $MSL10^{1-640}$-GFP was transiently transformed but mostly undetectable (Figure S3), indicating that the large deletion of the C terminus severely reduced its stability.

MSL10 is expressed in root tip, shoot apex and vascular tissues

Expression of MSL10 was earlier shown to be found in roots and leaves by MSL10p-GUS (Haswell et al. 2008). To further refine the expression pattern of MSL10, we examined more carefully on MSL10p-GUS reporter plants. Six out of 10 T1 transgenic lines showed similar expression patterns. GUS staining revealed that the expression is predominant in root

and shoot apices, which appear to correspond to their meristematic regions. Expression is also observed in the mid- and lateral veins of both young and old leaves (Figure S4).

DISCUSSION

To identify components involved in the wound signaling pathway, we performed a genetic screen using a wound inducible marker gene *RAP2.6*. A recessive mutant *rea1* was identified based on its ability to constitutively express *RAP2.6* gene. The *rea1* mutant displayed constitutive expression of *PDF1.2* and enhanced expression levels of *LOX2* and *AOS* after wounding. Consistent with its phenotype, *rea1* also accumulates a greater amount of JA than wild-type plants after wounding. Additionally, *rea1* exhibited a loss of apical dominance leading to a bushy phenotype. Apical dominance is thought to be caused by auxin (IAA) produced by the apical bud, which is transported basipetally from the apical bud, causing the lateral buds to remain in a dormant state. JA-responsive genes *LOX2*, *AOS* and *AtVSP* are found to be induced by IAA in *axr1-24* mutant (reviewed by Devoto and Turner 2003) and endogenous levels of auxins are also shown to decline after wounding, showing a significant cross-talk between the two hormone-dependent signaling pathways. The wild-type levels of JA in resting *rea1* mutant plants and the inability of the *coi1* mutation to completely suppress this phenotype strongly suggests that MSL10 may regulate additional pathways. Map-based cloning revealed that *REA1* encodes a mechanosensitive ion channel protein of small conductance and was earlier named as *MSL10* (Haswell and Meyerowitz 2006), attempt to complement *E. coli msc* mutants with *MSL10* was unsuccessful (data not shown). A serine to leucine ($MSL10^{S640L}$) substitution in the c-terminal end of the protein resulted in the gain-of-function phenotype of *rea1*. In addition to this, MSL10-GFP appears to be localized in the plasma membrane, but plants harboring this transgene phenocopied the *rea1* mutant, suggesting that the presence of a large GFP tag in the carboxy-terminus of *MSL10* interferes with its normal function.

Mechanosensitive ion channels have been identified in animals, plants and bacteria whose general function is predicted to be involved in sensing mechanical stimuli like sound, touch and osmotic pressure (Kung 2005; Perozo 2006; Arnadottir and Chalfie 2010), but direct evidence for their regulatory role during mechanical sensing in plants is lacking. MSL10 localizing in the plasma membrane suggests its involvement in an early signaling event. Loss of apical dominance, which is independent of JA accumulation and its signaling pathway and induced massive accumulation of JA, further implicate that *MSL10* might be involved in regulation of early events of wound signaling.

The *rea1* mutants show a constitutive cell death phenotype, which is mediated by COI1. This suggests that cell death in *rea1* mutant requires an intact JA signaling pathway and is independent of JA-hyperaccumulation, as untreated *rea1* mutant plants have wild-type levels of JA. Previous studies show that plants transiently produce reactive oxygen species (ROS) and superoxides in the damaged tissues (Li et al. 2012) and that this production is observed both locally and systemically (Orozco-Cardenas and Ryan 1999). However, application of MeJA has been shown to afford significant protection against xanthine/xanthine oxidase (X/XO) induced cell death (Overmyer et al. 2000). Therefore, cell death in *rea1*, which is presumably an enhanced phenotype of early wound response, requires JA signaling, but it is still not clear how JA acts as both a positive and negative regulator of cell death.

The important feature of the *rea1* mutant is its remarkable ability to accumulate JA after wounding. Mutants that overproduce JA were isolated and some of them have been cloned. Among them, *cev1*, *cet1* and *cet3* accumulate very high levels of JA constitutively, whereas the *fou2* mutant accumulates higher JA levels after wounding but the unwounded *fou2* leaves still have higher amounts of free JA compared to the wild-type plants. Contrary to this, *rea1* did not over-accumulate JA in untreated tissues. An interpretation for this could be that MSL10 is involved in the feedback regulation of JA synthesis.

rea1 phenotype is due to a mutation in a predicted mechanosensitive ion channel MSL10. When overexpressed in root protoplast and *Xenopus* oocytes, MSL10 exhibits MS ion channel activity (Haswell et al. 2008). The most extensive studied mechanosensitive ion channels are MscL (channel with large conductance) and MscS (Channel with small conductance) in *E. coli*. The *Arabidopsis* genome has 10 MscS-like genes (MSL) which are further subdivided into two classes based on the consensus sequence. Class I includes MSL1-3 that have the consensus sequence PF(X12-16)GXV(X20-21)PN(X9)N and has five trans-membrane domains. Class II includes MSL4-10 containing the consensus sequence F(X3)P(X3)GD(X10-14)V(X20-21)PN(X7)IXNXXR and has six trans-membrane domains. There are several closely homologous pairs indicating that they may be functionally redundant (Haswell 2007). MSL2 and MSL3 share an overall identity of 50% and the knockout of the individual genes did not have an observable phenotype. Whereas, the double mutants displayed a variegated phenotype with abnormal plastids, suggesting that these two could be functionally redundant (Haswell and Meyerowitz 2006). The lack of altered phenotype for *MSL10* T-DNA knockout lines further confirm that the $MSL10^{S640L}$ mutation is gain-of-function in nature. *MSL9* is the closest relative of *MSL10* and both of them share an overall identity of 62% and similarity of 76% at the amino acid level. However, *msl9*; *msl10* double mutants, *msl6*; *msl9*; *msl10* triple mutants (data not shown) and *msl4*; *msl5*; *msl6*; *msl9*; *msl10* quintuple mutant plants (Haswell et al. 2008) have no observable phenotype and respond normally to mechanical and osmotic stresses. Therefore, in addition to possible functional redundancy, we cannot rule out the possibility that MSLs in plants might be involved in a different physiological context.

The $MSL10^{S640L}$ is responsible for the severe phenotype of *rea1*. *MSL10-GFP* mimicked $MSL10^{S640L}$ phenotypes when overexpressed in WT plants, indicating that they function similarly. The previous patch clamp experiments performed in *Xenopus* oocytes showed that untagged MSL10 and MSL10-GFP have similar MS ion channel behavior (Maksaev and Haswell, 2012), making it less likely that $MSL10^{S640L}$ codes a misregulated ion channel. Furthermore, a recent publication showed that phosphorylated N-terminal domain of MSL10 induces cell death independent of MSL10 ion channel activity

(Veley et al. 2014). Overexpression of N-terminal truncation of MSL10 (1–136 amino acid) is capable of triggering cell death. Predicted by MSL10 topology, both N-terminus and C-terminus of MSL10 are at the cytoplasmic side. We hypothesize that the C-terminal domain of MSC10 normally stabilizes its N-terminus and prevents N-terminal induced cell death. S640L mutation or GFP tagging may disturb the C-terminal function of MSL10 thus leading to *rea1* phenotypes.

Although $MSL10^{S640L}$ is a gain-of-function mutation, it segregates recessively. Just like MscS forming heptamers, it is possible that this presumed MS ion channel of MSL10 forms homo-multimers. Since the wild-type monomers might have some suppressive effect, a channel with a mixture of mutant and wild-type monomers would be capable of functioning normally as shown by the WT/*rea1* heterozygotes and the complementation test. A channel only containing mutant monomers would result in *rea1* phenotype. However, overexpressing $MSL10^{S640L}$ or *MSL-GFP* in the wild-type plants gives high chance to form a channel simply made up of mutant monomers therefore produces *rea1* phenotype.

Wounding response involves perception, transduction of wound signals and activation of wound responsive genes. When operating under high levels of turgor pressure, the plasma membrane can sense these wound stimuli, resulting in the activation of ion channels. Therefore, mechanosensitive ion channels have an important role in wound signal perception and transduction. However, studying the function of individual mechanosensitive ion channels is difficult, as they are functionally redundant. Therefore, gain-of-function mutants like *rea1* not only aid in studying the wound inducible signaling pathway, but also act as a great tool for studying the functionally redundant genes like mechanosensitive ion channels.

MATERIALS AND METHODS

Plant culture conditions and treatments

Arabidopsis plants were grown on soil or Murashige and Skoog (MS) media at 20–23 °C under a photoperiod of 12 h of light and 12 h dark. Leaves were wounded mechanically by clipping with forceps and shock-frozen in liquid nitrogen at indicated times.

Identification of *rea1* mutant and luciferase activity assay

Homozygous RAP2.6-LUC transgenic seeds in Col-0 background (He et al. 2004) were mutagenized and 9,000 M_2 families were obtained as previously described (Shang et al. 2006). Approximately 16,000 M_2 plants derived from 300 pools were screened.

Four- to 6-week-old plants were sprayed with 1 mM lucuferin containing 0.01% Triton X-100 and the leaves were kept in dark for 5 min before the luminescence images were captured. Quantitative LUC assay was performed as described previously (He et al. 2004).

RNA extraction and Northern blot

Total RNA was isolated with TriZOL reagent (Invitrogen) according to the manufacturer's protocols. Twenty micrograms of total RNA was separated on formaldehyde gel transferred to nylon membranes and hybridized with [α-^{32}p] dCTP (Amersham) radio labeled probes.

Construct description

To generate the complementation construct P_{MSL10}:*MSL10*, the 6.3 Kb fragment including the whole ORF of *MSL10* and 2.0 Kb promoter region was digested with *Bcg*I (fill-in) and *Sal*I from BAC clone F14F18 (from ABRC) and subcloned into pCambia 1300 by *Hind*III (fill-in) and *Sal*I restriction sites. To obtain P_{35S}:*MSL10* and P_{35S}:$MSL10_{rea1}$, the coding region of *MSL10* was amplified from cDNA using primers 5′-CTAGGATCCTGGTAGTAGCATTGGAATG-3′and 5′-ACGCG TCGACTCAGTTCTTCTTTGTGAGATTAATG-3′ and cloned into *BamH*I and *Sal*I sites of pCambia1300:35S. The coding region was amplified with primers 5′-TACCTCGAGTGGTAGTAGCATTGGAATG-3′ and 5′-CC GTTCGAAGTTCTTCTTTGTGAGATTAATG-3′ and ligated to *Xho*I/*Csp45*I digested pER8-FLAG or pER8-GFP vectors to get *PXVE:MSL10-FLAG* or P_{XVE}:*MSL10-GFP*. The phenocopy construct P_{MSL10}:*MSL10-GFP* was obtained by *Afe*I/*Kpn*I digestion of the *MSL10* coding region and GFP sequence from P_{XVE}:*MSL10-GFP* and were ligated. The promoter region of *MSL10* was amplified with primers 5′-GGCAAGCTTCCAACAACAGTGTTATCGACTCATG-3′ and 5′-ATATCCATGGA AAGCAGAGCTGCAGAGGC GGTG-3′ and cloned into *Hind*III/*Nco*I digested pCambia1301 to construct P_{MSL10}:*GUS*.

Analysis of JA and OPDA

Jasmonic acid and OPDA extraction and quantification were carried out according to the protocol described in He et al. (2002).

Trypan blue staining

Leaves were soaked overnight in one volume of lactophenol and two volumes of ethanol, to remove chlorophyll. Lactophenol solution contains DL-lactic acid solution, glycerol, phenol and water of 1:1:1:1. The leaves were then changed into lactophenol solution with 1 mg/mL trypan blue for 10 min.

Protoplast transfection

Protoplasts were isolated from 6-week-old ecotype Columbia plants according to Sheen (http://genetics.mgh.harvard.edu/sheenweb/). 2×10^5 protoplasts were transfected with indicated constructs and incubated overnight in a 24-well microtiter plate before observed with Zeiss Meta 510 confocal microscope.

Histochemical GUS analysis

Seedlings at different stages were incubated overnight in 3 mM X-gluc and 100 mM sodium phosphate buffer, pH 7.0, with 0.1% (v/v) Triton X-100. Then chlorophyll was removed by washing the seedlings several times with 70% (v/v) ethanol.

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AUTHOR CONTRIBUTIONS

Y.Z. and S.C. performed most of the research and drafted the manuscript. P.H. carried out the genetic screen. H.F. and D.H. measured the levels of JA and OPDA. L.Y. performed RT-PCR analysis and quantified flower apices. M.S. analyzed the data. J.Z., X.T. and L.Z. designed the experiment, supervised the study, and revised the manuscript.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article at the publisher's web-site.

Figure S1. Positional cloning of gene responsible for *rea1* mutant phenotype

(A) Region of chromosome V to which *rea1* mutation was fine mapped to. Black boxes indicate the putative open reading frames within the BAC's (bacterial artificial chromosome) and the arrows indicate the direction of transcription. **(B)** Schematic diagram of predicted REA1 protein structure. The protein is composed of six transmembrane domains linked by cytosolic loops. The mutation in *rea1* at the carboxyl terminus of MSL10 is indicated by a red dot. **(C)** Predicted REA1 protein sequence. Sequence corresponding to transmembrane domains is underlined and the position of mutation is shown in bold.

Figure S2. Phenotypes of *rea1* mutant plants

(A) *MSL10* mRNA levels were detected by qPCR in the wild-type plants and the *rea1* mutants. The experiments were repeated three times. Unpaired *t*-test, Mean $\pm$ SD, $P = 0.041$. **(B)** Quantitative results of flowering apices in the wild-type plants and the *rea1* mutants. Unpaired *t*-test, Mean $\pm$ SEM, $n = 35$, $P < 0.0001$.

Figure S3. Membrane localization of MSL10 protein

Confocal images of protoplast cultures transformed with *MSL10-GFP*, *MSL10^{rea1}-GFP* and *MSL10$^{1-640}$-GFP* under the control of 35S promoter.

Figure S4. Expression pattern of *MSL10* gene Histochemical analysis of GUS activity in mature leaf

(A), young leaf **(B)**, seedling **(C)**, petiole **(D)** and roots **(E)** of *MSL10p-GUS* transgenic plants.

Table S1. Genetic analysis of *rea1* mutants

Plants were scored for both constitutive RAP2.6-LUC transgene expression and bushy phenotype.